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RESEARCH ARTICLE

Self-Assembled Phytosterol-Fructose-Chitosan Nanoparticles as a Carrier of Anticancer Drug

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Self-assembled nanoparticles were synthesized from water-soluble fructose-chitosan, substituted by succinyl linkages with phytosterols as hydrophobic moieties for self-assembly. The physicochemical properties of the prepared self-assembled nanoparticles were characterized by Fourier transform infrared spectroscopy, fluorescence spectroscopy, and transmission electron microscopy. Doxorubicin (DOX), as a model anticancer drug, was physically entrapped inside prepared self-assembled nanoparticles by the dialysis method. With increasing initial levels of the drug, the drug loading content increased, but the encapsulation efficiency decreased. The release profiles *in vitro* demonstrated that the DOX showed slow sustained released over 48 h, and the release rate in phosphate buffered saline (PBS) solution (pH 7.4) was much slower than in PBS solution (pH 5.5 and pH 6.5), indicating the prepared self-assembled nanoparticles had the potential to be used as a carrier for targeted delivery of hydrophobic anticancer drugs with declined cytotoxicity to normal tissues.

Keywords: Water-Soluble Chitosan, Phytosterol, Self-Assembled Nanoparticle, Anticancer Drug, Targeted Delivery.

1. INTRODUCTION

The critical bottleneck of conventional cancer chemotherapeutics includes severe cytotoxicity of most anticancer drugs due to indiscriminate distribution of drugs towards cancerous and normal tissues following systemic administration. In addition, anticancer drugs often suffer from poor solubility in water and thus require the addition of organic solvents or detergents for clinical applications, resulting in undesirable side effects such as venous irritation and respiratory distress.[1] Therefore, developing a distinct carrier system that incorporates a large quantity of drugs and specifically targets tumorous cells is indispensable for successful cancer chemotherapy. The application of nanotechnology in drug delivery has raised the expectations of achieving this goal and foreseeably changes the focus of the pharmaceutical and biotechnology industries in the future.

The interest in polymeric, self-assembled nanoparticles as anticancer drug delivery carriers is growing as a result of their promise in the following two respects. Firstly, they exhibit prolonged circulation time due to the nanoscale size and hydrophilic outer shell, which inhibit phagocytic and renal clearance. Secondly, selective accumulation of the polymeric nanoparticles in the cancerous tissues occurs because of the enhanced permeability and retention effect (EPR) induced by the defective vascular architecture of tumorous tissue and a poor lymphatic drainage system. In most cases, the polymeric nanoparticles consist of two parts: a hydrophobic core, which serves as the container for anticancer drugs, and a hydrophilic shell, which stabilizes the nanoparticles in aqueous environments. The anticancer drugs can be loaded into polymeric nanoparticles through two methods: physical entrapment or chemical conjugation. A hydrophobic interaction between the cores of the polymeric nanoparticles and the drug molecules allow the drugs to be entrapped in the polymeric nanoparticles.

Among different polymeric nanoparticles for targeted delivery of anticancer drugs, there has been rising interest in self-assembled nanoparticles based on natural polysaccharides, such as heparin,[2] dextran,[3] curdlan,[4] hyaluronic acid,[5] xyloglucan,[6] arabinogalactan,[7] alginate,[8,9] pullulan,[10–14] and chitosan.[15–21] In particular, chitosan has received increasing attention due to its specific structure and outstanding biological properties, such as biocompatibility, biodegradability, non-toxicity, and low immunogenicity.[22–24] Structurally, chitosan is very similar to cellulose, excepting the hydroxyl group in the C-2 position

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replaced by anamino group. The presence of the reactive amino group in chitosan offers great opportunity for chemical modification, which affords various chitosan derivatives for wider applications.[25–27]

Owing to the insoluble nature of chitosan ($pK_a = 6.4$) in water, the self-assembled nanoparticles from amphiphilic chitosan derivatives are easily precipitated in a biological solution. Therefore, water-soluble chitosan should be employed for fabrication of its self-assembled nanoparticles. In this study, self-assembled nanoparticles of phytosterol-fructose-chitosan (PFC) were synthesized with phytosterols as hydrophobic moieties. Phytosterols occuring in plants are cholesterol-like compounds, which can decrease serum cholesterol concentrations and reduce the risk of cardiovascular disease. Preliminary evidence indicates that phytosterols may help prevent ovarian, breast, prostate, colon, stomach, and lung cancer.[28] The physicochemical characteristics of the prepared self-assembled nanoparticles were studied using Fourier transform infrared spectroscopy, fluorescence spectroscopy, and transmission electron microscopy. Doxorubicin (DOX), an anticancer drug commonly used in the treatment of a wide range of cancers, was encapsulated in the prepared self-assembled nanoparticles by simple dialysis method, and its release characteristics were studied. The purpose of this work was to investigate the feasibility of phytosterols as hydrophobic moieties for preparing self-assembled nanoparticles and to evaluate self-assembled PFC nanoparticles as a potential carrier system for hydrophobic anticancer drugs.

2. EXPERIMENTAL DETAILS

2.1. Materials and Reagents

Chitosan (MW: 8×10^4) with approximately 85% deacetylation was purchased from Koyo Chemical Co., Ltd., Japan. Phytosterols were supplied by Xian Lantian Bioengineering Co.Ltd., China. Dicyclohexyl carbodiimide (DCC), 4-dimethylaminopyridine (DMAP), fructose, and pyrene were acquired from Sigma Chemical Co., USA. DOX hydrochloride (DOX · HCl) was obtained from Zhejiang Hisun Pharmaceutical Co. Ltd., China. Sodium borohydride was supplied by Sinopharm Chemical Reagent Co. Ltd., China. All other chemical reagents in the study were analytical grade and were obtained from commercial sources.

2.2. Synthesis of FC

The synthesis of FC consisted of two steps: chitosan was modified by fructose and then reduced by sodium borohydride.[29] Briefly, 8.87 g of fructose was added to 100 ml of 4.5% (w/v) chitosan solution prepared with 1% (v/v) acetic acid solution. Then the pH of the solution was adjusted to 4.5–5.0 using sodium hydroxide solution and stirred for 4 h at 37 °C. Next, 2.0 g of sodium borohydride was added to the stirred mixture and the pH of the polymeric solution was adjusted to 6.5–7.0 using hydrochloric acid solution. The reaction system was stirred for an additional 24 h and then the reaction was terminated by adding 95% alcohol. The precipitated polymer was filtered and washed three to four times with ethanol and diethylether, respectively. FC was dried in an infrared drier.

The solubilities of chitosan and FC in water were compared by preparing a series of 50 ml distilled water vials with 5 g chitosan or 5 g FC in each vial, respectively. Then all of the vials were gently stirred at 37 °C for the dissolution of solutes. The solubility of solutes in water was measured every 2 h by filtering and weighing the insoluble solutes.

2.3. Synthesis of Phytosterol Hemisuccinate

The synthesis was carried out according to a similar method previously described by Shaikh et al.[30] Briefly, 5.4 g of phytosterols were mixed with 3.6 g of succinic anhydride in 20 ml of pyridine. After allowing the reaction to occur for 24 h at room temperature, the mixture was precipitated in the ice diluted hydrochloric acid solution. The white powder of phytosterol hemisuccinate was obtained by recrystallization in tetrahydrofuran (THF) and ethanol.

2.4. Synthesis of PFC

Phytosterol hemisuccinate was coupled to FC by DCC/DMAP-mediated ester formation. Briefly, phytosterol hemisuccinate (300 mg) was socked in dried dimethyl sulfoxide (DMSO), and its carboxyl groups were activated by adding DCC (160 mg) and DMAP (80 mg) and magnetically stirring the mixture at room temperature for 2 h. Then, the activated phytosterol hemisuccinate was added to 50 ml of dried DMSO containing 1 g of FC and the reaction was allowed to occur at room temperature for 24 h. Subsequently, the reactant mixture was filtered and dialyzed against distilled water for 2 days using a dialysis tube (molecular cut-off: 12 kDa). Distilled water was changed at intervals of 3–6 h. The precipitated PFC was recovered by filtration, washed thoroughly with diethylether and distilled water, and lyophilized. To completely remove all non-reactants, the lyophilized PFC was redissolved in DMSO and dialyzed against distilled water. This process was repeated 3 times.

The schematic representation of the speculated synthetic route for PFC is shown in Figure 1.

2.5. Preparation of Self-Assembled PFC Nanoparticles

Self-assembled PFC nanoparticles with a roughly spherical shape were prepared by probe sonication in aqueous media. PFC was dispersed in distilled water with gentle shaking at 37 °C for 2 days, followed by sonication using a probe-type sonifier at 100 W for 2 min. The sonication

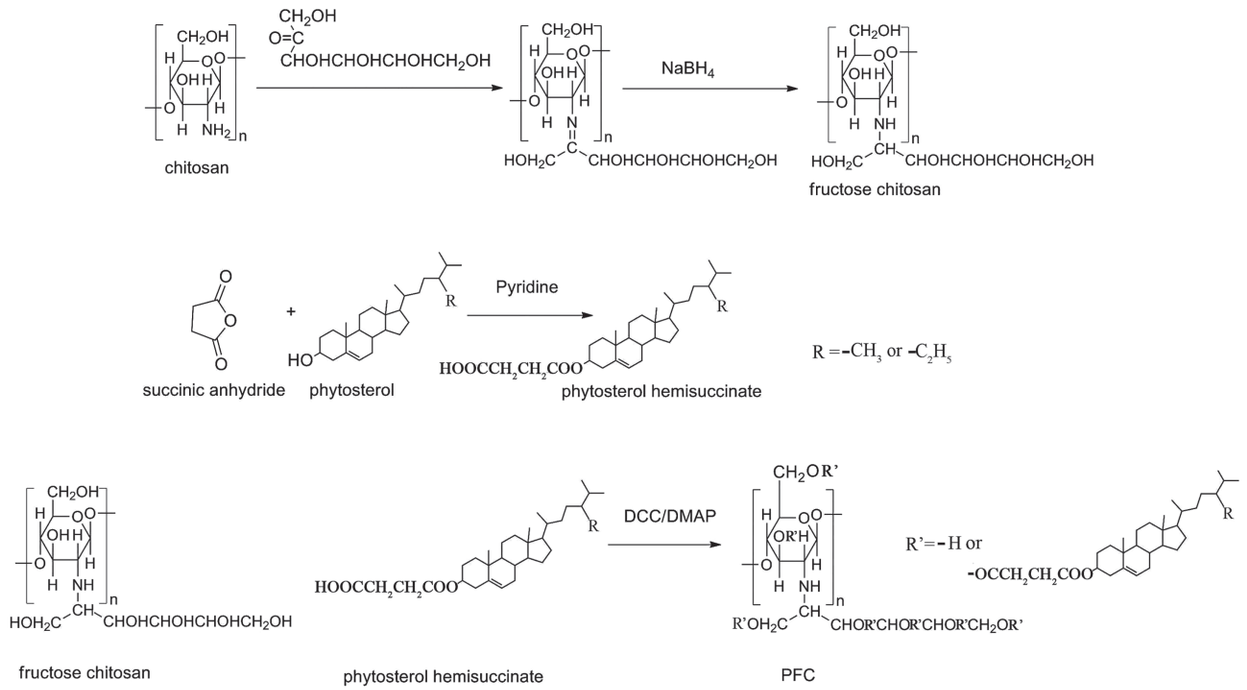

Fig. 1. Speculated synthetic route of PFC.

step was repeated three times until the desired size of PFC nonoparticles had been attained. To prevent heat built-up in the sample solution during the sonication, the pulse function (pulse on 2.0 s, pulse off 2.0 s) was used. The solution of self-assembled nanoparticles was then filtrated through a 1.0 μm Millipore filter to remove dust and impurities.

2.6. Fluorescence Spectroscopy of PFC

The self-aggregation behavior of PFC and its critical aggregation concentration (CAC) were investigated using fluorescence spectroscopy with pyrene as a hydrophobic fluorescent probe.[31] The pyrene solution (1.0×10^{-4} M) in acetone was added to a series of test tubes, which were then evaporated under a stream of nitrogen gas to remove the solvent. Then, PFC solutions of various concentrations were added to each test tube to bring the final concentration of pyrene to 6.0×10^{-7} M, which was nearly equal to the solubility of pyrene in water at 22 °C. The mixture solutions were sonicated for 30 min in an ultrasonic bath. Pyrene emission spectra were recorded using a fluorescence spectrophotometer (Hitachi F-4500, Hitachi Company, Japan). The probe was excited at 343 nm, and the emission spectra were obtained in the range of 360–420 nm at an integration time of 1.0 s. The slit width for excitation and emission were 10 and 2.5 nm, respectively. From the pyrene emission spectra, the intensity (peak height) ratio (I_3/I_1) of the third band (386 nm, I_3) to the first band (374 nm, I_1) was plotted against the logarithm of the PFC concentration. The CAC value was taken from the intersection of two straight lines.

2.7. Preparation of DOX Loaded Self-Assembled Nanoparticles

DOX, a hydrophobic anticancer drug, was physically entrapped in the prepared self-assembled nanoparticles by the dialysis method. Briefly, DOX · HCl (10 mg) was mixed with 3 equivalents of triethylamine in N,N-dimethyl acetamide (DMAc) (2 mL) to form the DOX basic adduct. Then a predetermined amount of PFC (200–500 mg) was added to the DOX solution, and the mixture was stirred overnight at 4 °C in the dark. The DOX/PFC mixture was then transferred to a dialysis tube (molecular cut-off: 12 kDa) and dialyzed against the phosphate buffered saline (PBS) solution (1/15 M, pH 7.4) for 3 days at room temperature. The buffer was changed with a fresh PBS solution every 4 h for the first 24 h, then daily. The DOX/ PFC mixture was filtered through a filter (1.0 μm, Millipore) to remove any polymer or drug that had precipitated out of solution, and then the solution was lyophilized to obtain the DOX-loaded nanoparticles.

2.8. Determining Loading Content (LC) and Encapsulation Efficiency (EE)

In order to measure the LC and EE of DOX, a lyophilized sample was suspended in DMAc and the solution was vigorously stirred for 2 h followed by 3 min of sonication. The resulting solution was centrifuged at $20000 \times$ g for 30 min, and the drug concentration in the supernatant was measured using a UV spectrophotometer (UV-2401PC,

Shimadzu, Japan) at 490 nm. Supernatant from the blank PFC nanoparticles was taken as correction.

LC is the percentage of DOX entrapped in PFC nanoparticles in relation to the amount of PFC. EE is the percentage of DOX loaded on PFC nanoparticles in relation to the amount of the initial amount of employed DOX. All samples were analyzed in triplicate. The LC and EE values were calculated according to the following equations:

$$\text{LC}\ (\%) = \frac{\text{Total amount of DOX in PFC}}{\text{Total amount of used PFC}} \times\ 100$$

$$\text{EE}\ (\%) = \frac{\text{Total amount of DOX in PFC}}{\text{Total amount of employed DOX}} \times\ 100$$

2.9. Release Profiles of DOX from Self-Assembled Nanoparticles *In Vitro*

DOX release behavior from self-assembled nanoparticles was studied *in vitro* by dialysis method against 1/15 M PBS solutions of pH 5.5, 6.5, 7.4, respectively. Briefly, 1.5 mL of DOX-loaded PFC nanoparticles (1 mg/mL) suspension was introduced into a dialysis tube (molecular cut-off: 12 kDa), and the dialysis tube was immersed in 30 mL of the three release media listed above at 37 °C with gentle shaking. At predefined time intervals, the release media was collected and fresh release media was added. The release amount of DOX was determined at 490 nm by UV method.

3. RESULTS AND DISCUSSION

3.1. Characteristics of FC and PFC

The solubilities of chitosan and FC in water are shown in Figure 2. It can be seen that chitosan is relatively insoluble in water, but FC shows an improved solubility up to 76% in water. This is due to the hydrophilic fructose moiety in FC, which decreased the interaction of hydrogen bonds between chitosan molecules.

The FTIR spectra of chitosan and PFC recorded on a FT-IR spectrometer (Tensor 27, Bruker Company, Germany) are shown in Figure 3. Compared with chitosan, it can be seen that the absorption band of carbonyl groups at 1665 cm^{-1} visibly increased in the FTIR spectrum of PFC. This can be used as an indirect evidence of phytosterol hemisuccinate introduced into fructose modified chitosan.

3.2. CAC of PFC

The fluorescence emission spectra of pyrene in PFC solution of various concentrations at room temperature are shown in Figure 4(A). The total emission intensity increased with as the PFC concentration increased. Notably, when the concentration reached the CAC, the third highest vibrational band at 386 nm (I_3) increased

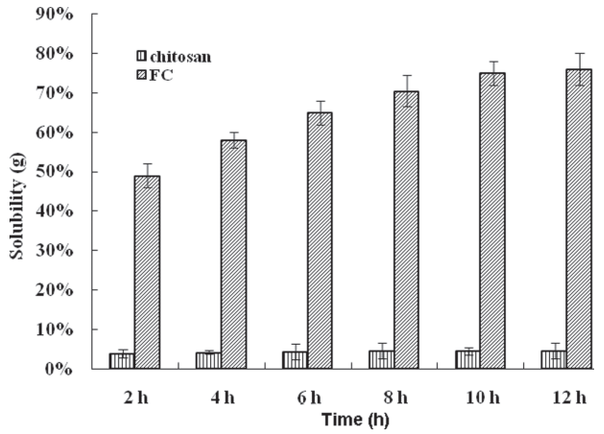

Fig. 2. Solubility of chitosan and FC in water.

drastically. However, any increase in the first band at 374 nm (I_1) was imperceptible, so the intensity ratio I_{386}/I_{374} was used to determine the CAC. Figure 4(B) shows that the CAC value of PFC was 0.036 mg/mL, which was significantly lower than those of certain hydrophobically modified chitosans, such as N-octyl-O-sulfate chitosan (0.45 mg/mL),[32] linolenic acid–modified chitosan (0.05 mg/mL),[33] and oligomer-conjugated glycol chitosan (0.041 mg/mL).[34] The low CAC value of PFC was one of the essential parameters for its use as carrier of hydrophobic drugs.

3.3. Self-Assembled Behavior of PFC

The self-assembled PFC nanoparticles of core–shell structure observed by transmission electron microscopy (Tecnai G2, FEI Company, American) are shown in Figure 5. TEM images show that self-assembled PFC nanoparticles are roughly spherical in shape with an average diameter of about 400 nm. The hydrophobic microdomains were formed by the association of the phytosterol moieties, and FC backbones coiled to form the hydrophilic shell outside these hydrophobic microdomains. Inter- and/or intramolecular hydrogen bonds packed FC backbones tightly, so the self-assembled PFC nanoparticles were relatively stable in aqueous media. This unique supramolecular structure is suitable for trapping hydrophobic drugs.

3.4. LC and EE of DOX in PFC Nanoparticles

Ideally, a successful drug carrier system should have high EE for loaded drugs. Since loaded antitumor drugs are very expensive, low EE causes waste and limits the use of such carrier systems. The LC and EE of DOX in PFC nanoparticles are shown in Table I. It can be seen that by decreasing the ratio of drug to carrier, the LC of DOX in PFC nanoparticles decreased while the EE of DOX in PFC nanoparticles increased. The EE of DOX in a PFC carrier reached 85% when the weight ratio of DOX to polymer was 1/50.

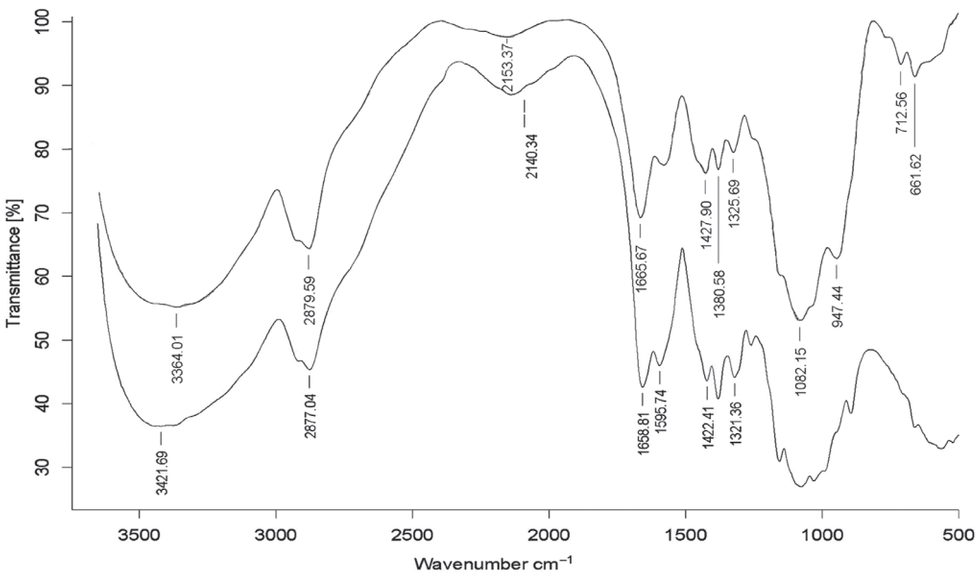

Fig. 3. FTIR spectra of chitosan (below) and PFC (above).

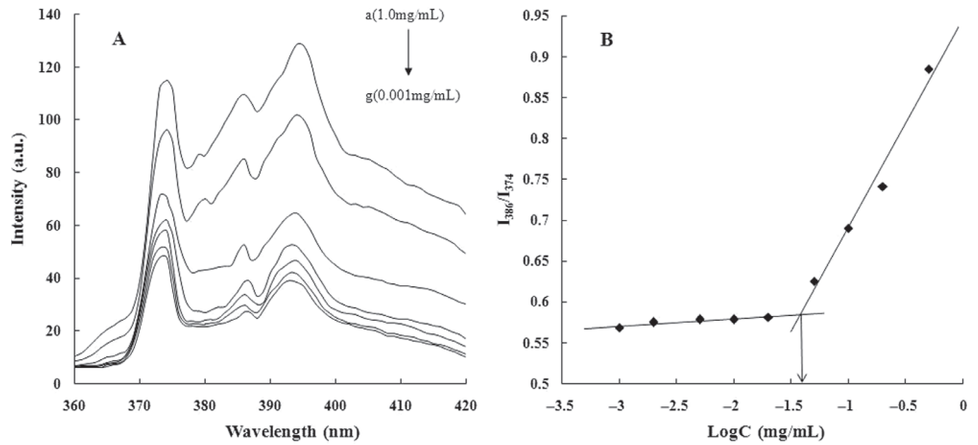

Fig. 4. Fluorescence emission spectra of pyrene in PFC solutions with various concentrations (A) and intensity ratio (I_{386}/I_{374}) from the pyrene emission spectra as a function of PFC concentration (B).

3.5. DOX Release Behavior *In Vitro*

PBS solution (pH 7.4) was used as release medium for studying the DOX release character of DOX-loaded PFC nanoparticles with different ratios of drug/carrier (1/30, 1/40, and 1/50). Three DOX-loaded PFC nanoparticles exhibited sustained release profiles. DOX release rate decreased evidently with the increase of drug loading content (Fig. 6). This result suggests that an increase in the amount of DOX enhanced the inner core hydrophobicity of self-assembling nanoparticles, which resulted in slower drug release. It is also possible that the hydrophobic drugs crystallized in the inner-core of the self-assembling nanospheres. Crystallized drugs are expected to dissolve and diffuse more slowly to the release media at higher levels of drug-loading contents, which could lead to slow diffusion into the outer aqueous phase. Therefore, DOX release from the PFC nanoparticles with higher drug contents showed slower release rate than the PFC nanoparticles with lower drug contents.

Table I. LC and EE of DOX in PFC nanoparticles.

Sample	Drug/carrier[a]	DOX-loaded particle size[b]	LC (100%)[c]	EE (100%)[d]
DOX_0	1/20	292 ± 13	3.0 ± 0.14	61 ± 1.2
DOX_1	1/30	320 ± 19	2.3 ± 0.6	69 ± 0.72
DOX_2	1/40	354 ± 27	1.9 ± 0.41	77 ± 0.34
DOX_3	1/50	379 ± 32	1.7 ± 0.3	85 ± 0.56

Notes: [a]DOX/PFC self-aggregated nanoparticles (mg/mg); [b]Diameter of particle (mean value ± SD) measured by dynamic light scattering; [c](Loading DOX/PFC self-aggregated nanoparticles) · 100% (mean value ± SD) determined by spectrophotometric method with three times. [d](Loading DOX/total DOX) · 100% (mean value ± SD) determined by spectrophotometric method with three times.

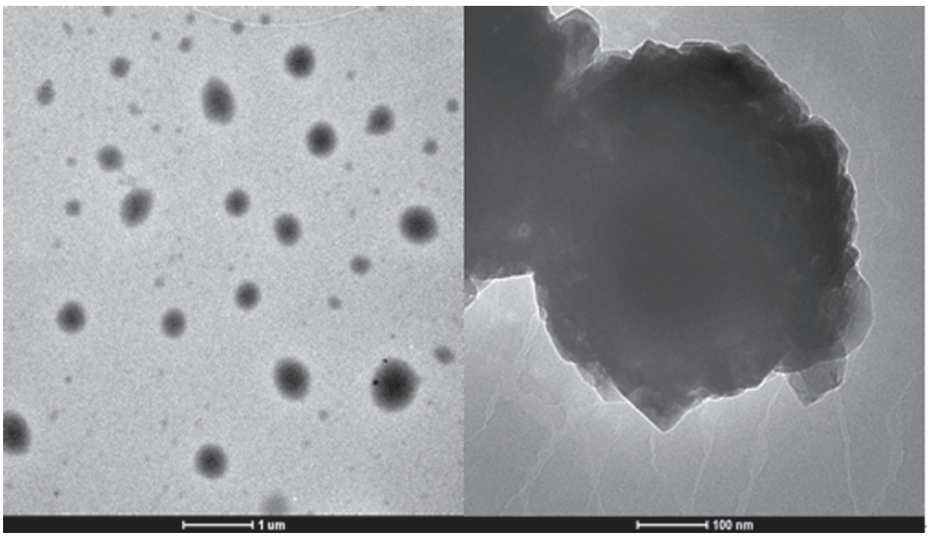

Fig. 5. TEM images of self-assembledPFC nanoparticles, where the scale bars are 1000 nm and 100 nm for left and right, respectively.

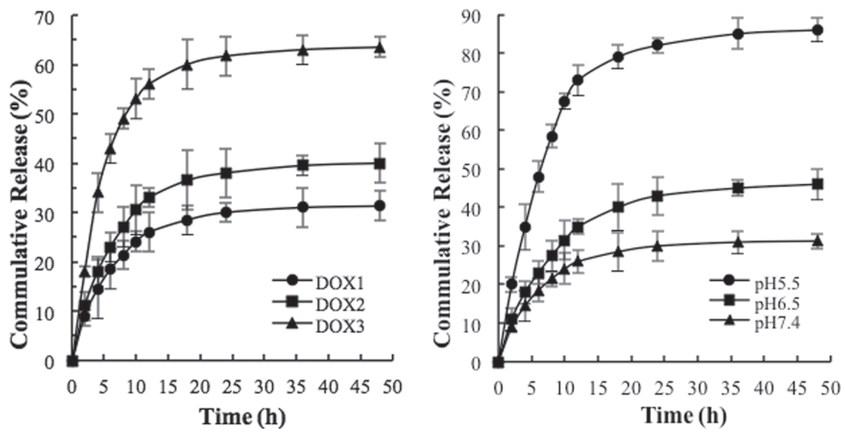

Fig. 6. Release behavior of DOX in PBS solution of pH 5.5 (left) and DOX_1 in PBS solutions with different pH values (right).

In order to simulate the physiological environment, three PBS solutions with different pH values (pH 7.4, 6.5, and 5.5) were used as release media to study the DOX release character of DOX-loaded PFC nanoparticles (DOX_1). The DOX-loaded PFC nanoparticles exhibited release profiles related to the pH value of the release media. As shown in Figure 6(B), DOX release rate decreased with the pH increase of the release media, and similar release characteristics were also discovered in other DOX-loaded samples with different drug loading contents (data not shown). This was mainly due to the following two factors. Firstly, as a weak base, the solubility of DOX is greatly influenced by the pH of the aqueous media, and its solubility in the acidic media was higher than in the alkaline media. Therefore, DOX released from self-assembled PFC nanoparticles much faster at pH 5.5 and 6.5 than at pH 7.4. Secondly, PFC is also a pH sensitive polymer because of the presence of amino groups in chitosan molecules. When the pH of the aqueous media decreased, the self-assembled PFC nanoparticles absorbed water to swell and induced their conformational change and structural disintegration, which accelerated drug release from self-assembled PFC nanoparticles.

The extracellular pH of normal tissues and blood pH were kept constant at pH 7.4. However, the pH of most tumorous tissues is lower than normal tissues. DOX was released very slowly at pH 7.4, and the total release amount was about 31.3% in 48 h. This release characteristic of DOX suggests that the self-assembled PFC nanoparticles have the potential to be used as a carrier for sustained release of hydrophobic anticancer drugs while posing declined cytotoxicity to normal tissues.

4. CONCLUSIONS

A novel amphiphilic polymer, PFC, which formed monodisperse, self-assembled nanoparticles in aqueous media, was successfully synthesized. The anticancer drug, DOX,

was loaded in core–shell structure of self-assembled PFC nanoparticles by a simple dialysis method with high EE. The release profiles *in vitro* demonstrated that the release rate of DOX in PBS solution (pH 7.4) was much slower than in PBS solution (pH 5.5 and pH 6.5), which indicated that DOX-loaded PFC nanoparticles could reduce the cytotoxicity to normal tissues. These results clearly suggest that self-assembled PFC nanoparticles have excellent potential as a sustained release carrier of hydrophobic anticancer drugs.

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